Abstract

Eukaryotic cells have developed elaborate biochemical processes to counteract oxidative stress, electrophiles, or carcinogens due to environmental insults. One of these important cellular defense mechanisms is mediated by the transcription factor Nrf2 that have been shown to have decrease activity with age. While the process of which Nrf2 is involved with has been extensively studied, its exact mechanism for activation is still unclear. Using HepG2 cell cultures, nuclear extraction techniques, SDS-PAGE, Western Blotting, Chemical Inhibitors, and EMSA, we showed that the MAP Kinase family is involved in the activation of Nrf2. Future direction will be towards determining exactly which proteins in the MAP Kinase family is involved in this activation and whether or not it has decrease activity with age.

Activation and Nuclear Translocation

of the Transcription Factor Nrf2

by the MAP Kinase Family

By

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Preface

Eukaryotic cells are constantly exposed to oxidative stress, electrophiles, or carcinogens due to environmental insults. To counteract these effects, our bodies have developed elaborate biochemical processes that help us deal with these harmful effects and it starts within our cells. One of these important cellular defense mechanisms is mediated by the transcription factor Nrf2. Significant research has been toward deciphering the effects of Nrf2 on cellular stress coping mechanisms but the exact signal transduction pathway leading to the induction of Nrf2 is still unclear. This research is focused on uncovering the enzymes that are involved in the activation of Nrf2 that allows it to participate in further stress coping mechanisms.
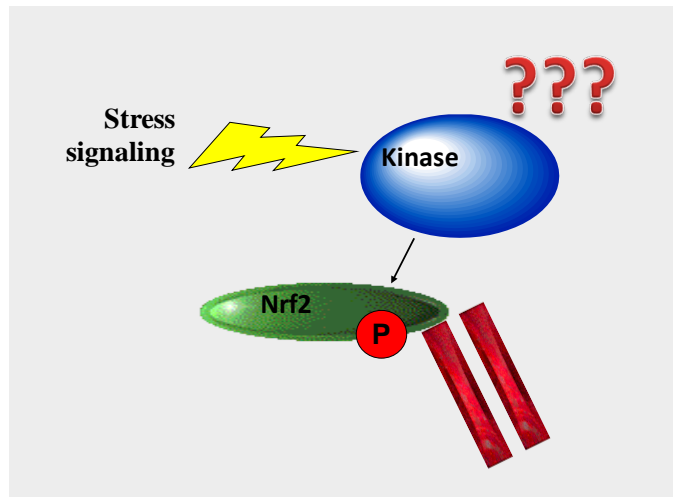

Figure 1. **Project Objective.** Studying which compound is involved in the induction of Nrf2, thus allowing it to participate in further signal transduction pathways.

Background

Nrf2 and Its Significance

Nrf2 was first discovered through the search for an anti-carcinogenic compound. The first concept of chemoprevention came from the observation from Richardson in 1951 that a systemic administration of small amounts of xenobiotics decreased the incidence of cancer in rats that were fed large doses of carcinogenic azo dyes.[1] Further research resulted in a number of chemopreventive compounds that had these anti-carcinogenic qualities and this was due to their ability to induce expression of phase II enzymes and endogenous antioxidants that defend cells from oxidative stress or reactive carcinogenic intermediates. [2, 3, 4] A similarity between these phase II enzymes is a promoter region containing specific DNA sequences that are called the antioxidant response elements (AREs) or the electrophile response elements (EPREs).[5] Efforts to find the transcription factor that binds to the AREs that lead to the transcription, subsequent translation and activity of key antioxidant enzymes resulted in the discovery of the transcription factor NF-E2-related factor 2 (Nrf2).[6] This transcription factor proved to be a key modulator of the expression of phase II detoxification

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[1] Richardson, H. L., Cunningham, L. (1951). The inhibitory action of methylcholanthrene on rats fed the azo dye 3'-methyl-4-dimethylaminobenzene. *Cancer Res.* 11:274.

[2] Kensler, T. W., Curphey, T. J., Maxiutenko, Y., Roebuck, B. D. (2000). Chemoprotection by organosulfur inducers of phase 2 enzymes: dithiolethiones and dithiins. *Drug Metabol. Drug Interact.* 17:3–22.

[3] Dinkova-Kostova, A. T., Holtzclaw, W. D., Cole, R. N., Itoh, K., Wakabayashi, N., Katoh, Y., Yamamoto, M., Talalay, P. (2002). Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc. Natl. Acad. Sci. USA* 99:11908–11913.

[4] Talalay, P., Fahey, J. W. (2001.) Phytochemicals from cruciferous plants protect against cancer bymodulating carcinogen metabolism. *J. Nutr.* 131:3027S–3033S.

[5] Jeyapaul, J., Jaiswal, A. K. (2000). Nrf2 and c-Jun regulation of antioxidant response element (ARE)-mediated expression and induction of gamma-glutamylcysteine synthetase heavy subunit gene. *Biochem. Pharmacol.* 59:1433–1439.

[6] Venugopal, R., Jaiswal, A. K. (1996). Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. *Proc. Natl. Acad. Sci. USA* 93:14960–14965.

enzyme and endogenous antioxidants.[7] Nrf2 regulates a variety of genes in addition to the classical phase II enzymes. It has been shown to help regulate cellular redox homeostasis, cell growth and apoptosis, inflammatory response and the ubiquitin-mediated degradation pathway.[8] These pathways are together called an Nrf2-mediated defense response and are crucial for cells to counteract the harmful environmental insults and to maintain cellular redox homeostasis. Although the search for an anti-cancer agent led to the discovery of Nrf2, reactive oxygen species have been linked to pathogenesis of many diseases other than cancer. Nrf2 could be the key in many other pathological conditions, such as Alzheimer's disease, Parkinson's disease, ischemia, aging and cardiovascular disease, pulmonary fibrosis and acute pulmonary injury, inflammation, emphysema, asthma, lupus-like autoimmune nephritis, and macular degeneration. [7]
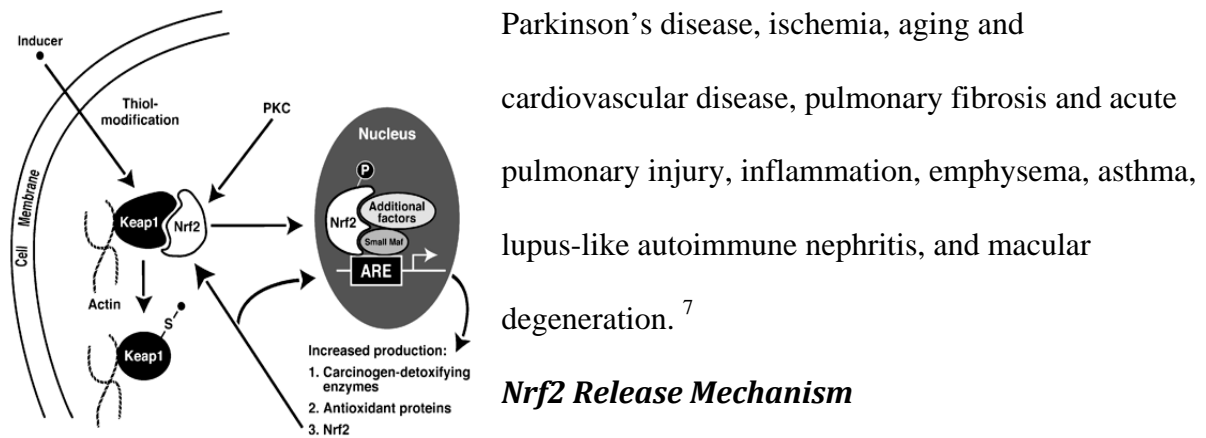
Figure 2. **Keap 1 and Nrf2 release mechanism.** Nrf2 is bound to a negative regulator names Keap1. Keap1 is a cytoplasmic factor that binds to actin cytoskeleton and Nrf2 to keep Nrf2 in the cytoplasm. Upon exposure to oxidative stress or chemopreventive compounds, Keap1 releases Nrf2, upon dissociation from Keap1, Nrf2 translocate to the nucleus, forms a heterodimer with Maf, and ultimately activates ARE-dependent gene expression.

Nrf2 Release Mechanism

Figure 2 below is a general diagram for the signal transduction pathway that activates the transcription of antioxidant enzymes. Nrf2 is the transcription factor for this pathway; normally, Nrf2 is bound in the cytoplasm by KEAP1, an actin binding protein attached to the cytoskeleton. When the cell encounters a stress, a kinase is activated and it in turn phosphorylates Nrf2, this allows

[7] Zhang, Donna D.(2006)'Mechanistic Studies of the Nrf2-Keap1 Signaling Pathway', Drug Metabolism Reviews,38:4,769 — 789

[8] Thimmulappa, R. K., Mai, K. H., Srisuma, S., Kensler, T. W., Yamamoto, M., Biswal, S. (2002). Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer Res.* 62:5196–5203.

Nrf2 to dissociate from KEAP1 and enter the nucleus. A kinase is a type of enzyme that transfers phosphate groups from high-energy donor molecules, such as ATP, to specific target molecules called substrates, in this case, Nrf2. The transfer of a phosphate group to a protein or a small molecule is called phosphorylation. The phosphorylation of Nrf2 has been suggested by Bloom and Jaiswal[9] to induce a conformational change that prevents further attachment to Keap1 and relocate to the nucleus. Once Nrf2 is in the nucleus it binds to the Antioxidant Response Element, abbreviated ARE, and promotes transcription of glutathione synthesizing genes. The ARE is a specific enhancer found in the promoter of genes for glutathione synthesizing and other phase II enzymes. The transcription factor Nrf2 has been implicated as the central protein that interacts with the ARE to activate gene transcription in response to an oxidative stress signal.

In the search to study how Nrf2 dissociates from Keap1, many investigators have discovered that phosphorylation of Nrf2 regulates its activity. Kinases that have been reported to have the potential to facilitate this phosphorylation include protein kinase C (PKC); PERK, an endoplasmic reticulum (ER)-transmembrane protein kinase that signals ER stress; the kinases in the MAP kinase pathway, such as extracellular-regulated kinase (ERK), P38, Jun N-terminal kinase (JNK); and phosphatidylinositol 3-kinase (PI3K).[10] Our research aims to determine which one of these kinases is directly involved in the phosphorylation of Nrf2 that leads to its dissociation from KEAP1 and its subsequent nuclear translocation. This was done through the systemic administration of known oxidative stress inducers: acetaminophen and menadione.

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[9] Bloom, DA and Jaiswal, AK. J Biol Chem. 2003 Nov 7;278(45):44675-82. Epub 2003 Aug 28.
Figure 2. Gene Logic Inc. Oxidative Stress Induced Gene Expression Via Nrf2. Accessed May 2009. < http://owl.english.purdue.edu/owl/resource/557/09/>
[10] Kong et al. Induction of xenobiotic enzymes by the MAP kinase pathway and the antioxidant or electrophile response element (ARE/EpRE). Drug Metab Rev. 2001 Aug-Nov;33(3-4):255-71.

MAP Kinase Family and Its Significance

MAP kinase family proteins are highly conserved signaling kinases that act to regulate cell growth, differentiation, and stress responses.[11] Within the MAPK family, there are three distinct families of MAP in mammalian cells, these are the p42/44 extracellular signal-regulated kinase (ERK) MAPKs, c-Jun NH2-terminal kinases (JNKs), and p38 MAPK.[12] SAPK/JNK has been shown to play a significant role in a number of cellular responses against oxidative stress, one of which is DNA damage. [13] ERK, P28 and SAPK/JNK play a key role in apoptosis as well as responding to reactive oxygen species.[14] There are many neurodegenerative disorders that are, in part, a result of apoptosis mediated by an irregular flux of key kinases, such as Parkinson's disease[15], Alzheimer's disease[16], and Huntington's disease[17].

Induction of Nrf2 – Acetaminophen and Menadione

Acetaminophen, shown in Figure 3 on the left, [N-acetyl-p-aminophenol (APAP)] is one of the most widely used analgesic and antipyretic drugs worldwide under the brand name of Tylenol. In high dosage, sulfation and glucuronidation become saturated and glutathione (GSH) is depleted by the reactive intermediate N-acetyl-p-benzoquinoneimine (NAPQI). Excess of this reactive intermediate causes oxidative stress and binds covalently to liver proteins. This in turn results in adverse cellular affects, depresses mitochondrial functions,

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[11] Cobb, M.H., and Goldsmith, E.J. 1995. How MAP kinases are regulated. J. Biol. Chem. 270:14843–14846.

[12] Lee, J.C., *et al.* 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature.* **372**:739–746.

[13] Kufe et al. Translocation of SAPK/JNK to mitochondria and interaction with Bcl-xL in response to DNA damage.THE JOURNAL OF BIOLOGICAL CHEMISTRY. Vol. 275, No. 25, Issue of June 23, pp. 19433–10424, 2000

[14] Yeste-Velasco, et al. The p38MAPK signaling pathway regulates neuronal apoptosis through the phosphorylation of the retinoblastoma protein. Neurochemistry International 54 (2009) 99–105

[15] Alvira, D., Ferrer, I., Gutierrez-Cuesta, J., Garcia-Castro, B., Palla` s, M., Camins, A., 2008. Activation of the calpain/cdk5/p25 pathway in the girus cinguli in Parkinson's disease. Parkinsonism Relat. Disord. 14, 309–313.

[16] Thakur, et al., 2008. Retinoblastoma Protein phosphorylation at multiple sites is associated with neurofibrillary pathology in Alzheimer disease. Int. J. Clin. Exp. Pathol. 1, 134–146.

[17] Pelegrı´, et al. 2008. Cell cycle activation in striatal neurons from Huntington's disease patients and rats treated with 3-nitropropionic acid. Int. J. Dev. Neurosci., August 12.

and disrupts calcium homeostasis and redox balance.[18] Menadione, shown in Figure 3 on the right, induces reactive oxygen species generation via redox cycling which has been shown to promote apoptosis of murine pancreatic acinar cells. [19] The pathways in which detoxification of these compounds occurs is through a Nrf2 mediated pathway and it is therefore effective to induce Nrf2 nuclear translocation.[20]
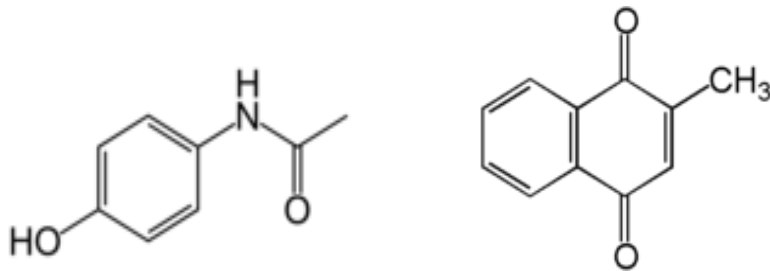

Figure 3. **Tylenol (left) and Menadione (right) structure.** Shows the structure of the two compounds that was used in our in experiments to induce Nrf2 nuclear translocation.

The effects of APAP and Menadione on our cells were studied treating the HepG2 cells with the compounds at various concentrations and time frames, lysing the cells, running SDS-PAGE that denatures the proteins and runs them through the gel according to size. The results are then viewed by binding a specific antibody to Nrf2 through a western blotting procedure along with a tagged secondary antibody and chemiluminescent reagent to observe the concentration of Nrf2 in the nucleus. In order to confirm that Nrf2 does indeed bind to the ARE once it enters the nucleus, we ran an Electrophoretic Mobility Shift Assay (EMSA), both the lightshift and supershift. The EMSA is because DNA-protein complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis.

[18] Cohen, S. D., Hoivik, D. J. & Khairallah, E. A. (1998) in *Toxicology of the Liver*, eds. Plaa, G. L. & Hewitt, W. R. (Taylor & Francis, Bristol, PA), Vol. 1, pp. 159–186.
[19] J. Biol. Chem., Vol. 281, Issue 52, 40485-40492, December 29, 2006
[20] Chan et al. An important function of Nrf2 in combating oxidative stress: Detoxification of acetaminophen. PNAS (89-8): 2001 pg 4611–4616

Experimental Procedure

HepG2 Cell Cultures. Human liver carcinoma cell line (HepG2) cells were grown in Modified Eagle's Medium with 10% Fetal Bovine Serum, 5 mL l-glutamine, and 5 mL of antibiotic solution (Sigma-Aldrich A5955). Cells were allowed to grow on 6 well, 60 mm each plates to the desired density and then treated with either acetaminophen solution (5 mM) or menadione (50 µM). After the allocated time, the cells were harvested by first vacuuming off the media, washing gently with cold PBS, and scraping into a 1.5 mL eppendorf tube and put on ice. Cells were centrifuged at 2000 rpm and the supernatant was removed. Pellets were frozen for future use.

Kinase Inhibitors. Cells were pretreated with kinase inhibitors and incubated for 2 hours before adding APAP or menadione. Inhibitors included U0126, a pan-MAPK inhibitor (10 µM); H-89 (EMD #171260), a PKA inhibitor (30 µM); and Compound C (EMD, #171260), an AMPK inhibitor (20 µM).

Nuclear Extraction. Cell pellets were resuspended in 100 µL of hypotonic lysis buffer (10 mM HEPES, pH 7.9, with 1.5 mM MgCl2 and 10 mM KCl) and incubated for 15 minutes on ice. Six µl of 10% IGEPAL CA-630 was added to each tube for a final concentration of 0.6%, then vortexed vigorously for 10 seconds. The extract was then centrifuged at 10,000 rpm for 1 min in at 4ºC. The cytoplasmic supernatant was transferred into an eppendorf tube and the nuclear pellet was resuspended in 100-µL extraction buffer (20 mM HEPES, pH 7.9, with 1.5 mM MgCl2, 0.42 M NaCl, 0.2

Figure 4. **SDS PAGE contraption.** Shown above is the set up used to separate the proteins by charge distribution.

mM EDTA, and 25% (v/v) glycerol) containing protease inhibitor cocktail. The suspension

was then sonicated to fragment the DNA and placed in the freezer for future use.

 SDS-PAGE. Lysate was thawed and 30 µg of protein was mixed with 2X Laemmli loading

buffer and boiled for 5 minutes before running on a 7.5% polyacrylamide gel, Figure 4 shows

the contraption used to run the SDS-PAGE. Proteins were transferred onto PVDF membrane.
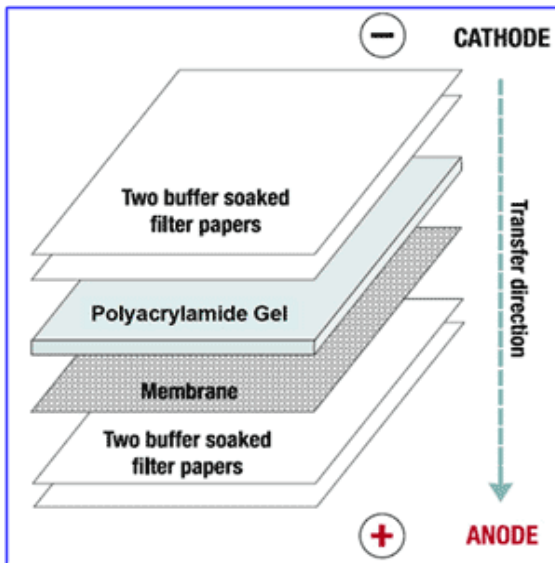

Figure 5. **Western Blotting.** Diagram to show the general theory of western blotting where the current induces the migration of proteins from the polyacrylamide gel into the membrane.

Western Blotting Analysis. **(shown in Figure 5 below)** Membranes were blocked in a blocking buffer containing 1% Tween-20 and 5% nonfat dry milk, incubated with primary antibodies for 2 h at room temperature, washed with PBS-Tween, and incubated with secondary antibodies for 1 h at room temperature. Following an additional wash, the membranes were incubated with [21]chemiluminescence reagents (Pierce Pico West), exposed to film, and developed.

Antibodies made to the following proteins were used at 1:1,000 dilutions: Nrf2 (H-300 from Santa Cruz Biotech), Actin (A5441 Sigma-Aldrich), and various MAPK antibodies (Cell Signaling Technology No. 9926). Blots were densitometrically analyzed with ImageJ from NIH.

Electrophoretic Mobility-Shift Assay (EMSA). Transcription factor binding to the ARE was determined by using an EMSA. Nuclear extracts were prepared as described. All gel-shift assays were performed for three sample replicates in each group. Using a synthetic

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[Figure 5.] Fermentas. Semi Dry Protein Transfer for Western Blotting. Accessed: May 2009. < http://www.fermentas.com/techinfo/electrophoresis/pproteintransfer.htm>

double-stranded oligonucleotide probe for the ARE (5′-TGG GGA ACC TGT GCT *GAG*

TCA CTG GAG-3′) (Santa Cruz Biotechnology). Binding reactions containing equal

amounts of protein (9 μg) and labeled oligonucleotide probes were performed for 20 min at

room temperature in binding buffer (4% glycerol/1 mM $MgCl_2$/0.5 mM EDTA/0.5 mM

DTT/50 mM NaCl/10 mM Tris, pH 8.0). Specific binding was confirmed by using 100-fold

excess unlabeled ARE oligonucleotide as a specific competitor. Protein–DNA complexes

were separated by gel electrophoresis by using 6% nondenaturing polyacrylamide gels

followed by transfer to nylon membrane and UV cross-linking to detect the degree of

retardation produced by binding to the probe, according to the procedure from Pierce

Lightshift Chemiluminescent EMSA kit.

Supershift Assay. Binding of Nrf2 to the ARE was determined by supershift assays where

anti-Nrf2 antibodies were incubated with the nuclear extracts at 4°C overnight before

carrying out the EMSA reaction.

Results and Discussion[22]

Determining Test Conditions

Using the methods described in the experimental procedure section above, the optimum

conditions to induce Nrf2 nuclear translocation were determined. Shown Graph 1 is the

western blot of nuclear Nrf2 under various treatments and time, the intensity of the blot

shows the amount of Nrf2 in the nucleus; the darker the blot, the more Nrf2. The conditions

were APAP 1 hr, Menadione 4 hrs, APAP 4 hrs in serum-free medium, APAP 1hr,

Menadione 4 hrs, and APAP 4 hrs (all conditions were carried out in serum (FBS)-containing

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[Note] Please note that in the Graphs, there are differences of scales. This is because each blot scanned individually and the units are arbitrary. No comparisons between Graphs are warranted. They are showing relative values so one can only make comparisons within a Graph, not between multiple Graphs.

media except as noted). From this data, the conditions that best induced Nrf2 to localize into the nucleus are APAP 1 hr in serum-free and Menadione 4 hrs in complete media.
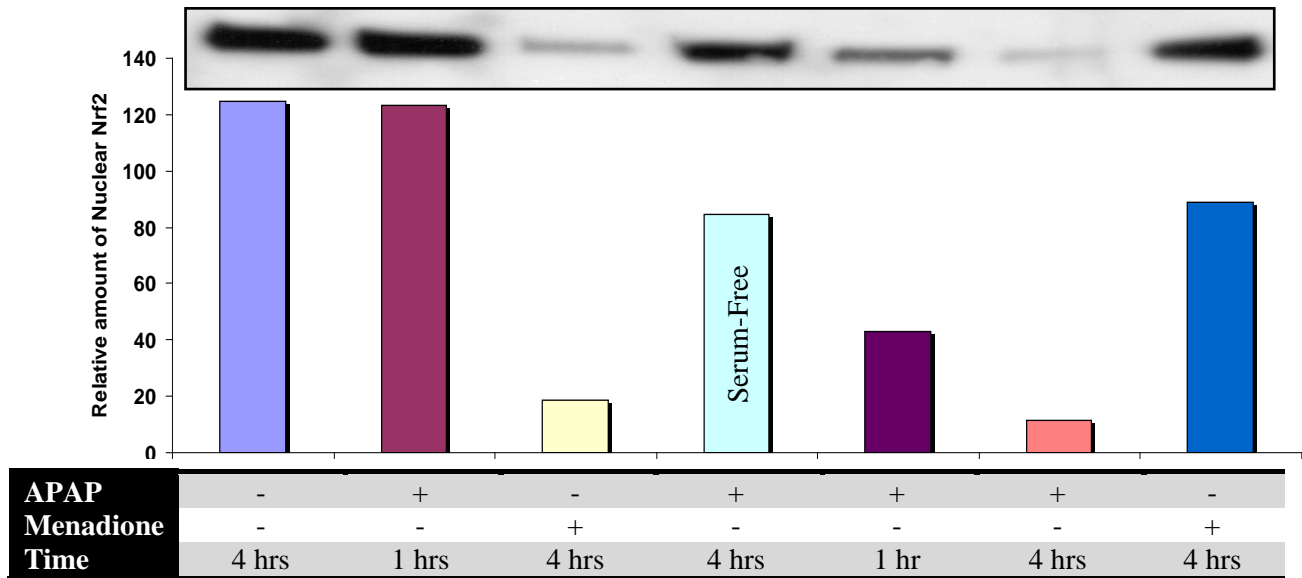
Graph 1. **Testing effect of APAP and Menadione on Nrf2 nuclear localization.** Western blot of nuclear Nrf2 under various treatments and time, the intensity of the blot shows the amount of Nrf2 in the nucleus.

Figure 6 shows the western blot for additional conditions using Serum vs. Serum-Free medium. From this data, it appears that treatments with serum interfere with cellular uptake of APAP but not of menadione. This could be due to APAP binding with the growth factors and proteins in the media before it even enters the cells. In order to account for these effects, our ideal test conditions are APAP 1 hr in serum-free and Menadione 4 hrs in serum. In both of these experiments, our vehicle control has unexpectedly high Nrf2 levels. We did not continue to have this problem in later experiments.
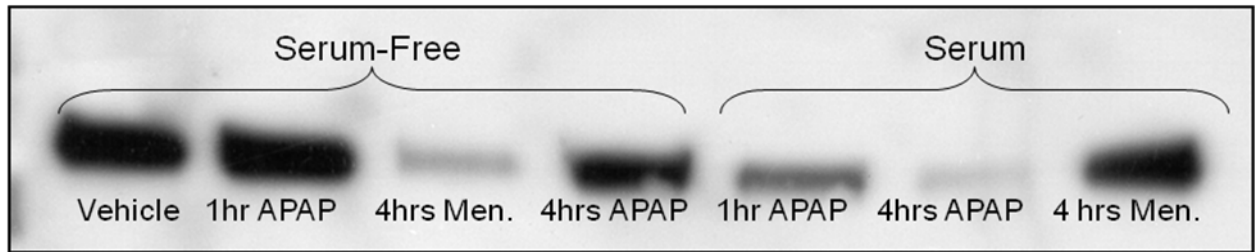
Figure 6. **Testing Serum vs. Serum-Free.** Western blot for conditions using Serum vs. Serum-Free medium indicating that APAP is effected by serum in the medium.

MAP Kinase Family

In order to determine if the MAPK Kinase family is involved in the transcription of Nrf2 nuclear translocation and thus antioxidant synthesis, we used a MAPK Kinase family inhibitor called UO126 that inhibits the phosphorylation, therefore activity, of all the kinase in the MAPK Kinase family. From previous experiments, cells under the conditions that best induce Nrf2 nuclear localization were pretreated with UO126 to see if there is a decrease in the levels of Nrf2 in the nucleus. Shown in Graph 2 is the western blot of nuclear Nrf2 when the cells are treated with the MAPK Kinase inhibitor, UO126 before APAP or menadione. From this data, the vehicle with no treatment has low levels of nuclear Nrf2 as expected; with the addition of UO126, it is even lower although this is not statistically significant. Also on the Graph are the two conditions that are known to best induce Nrf2 nuclear localization and the same condition with the MAPK Kinase family inhibitor, UO126. The menadione 4 hrs in serum and menadione 4 hrs in serum with UO126 showed a decreased in nuclear Nrf2 when cells are treated with inhibitor. The APAP 1 hr in serum-free and the APAP 1 hr serum-free with UO126 also showed a decrease in levels of nuclear Nrf2 with inhibitor. Three trials were run for these conditions with a p value of < 0.05 for the menadione with and without UO126. We can conclude that the MAPK Kinase family does induce Nrf2 nuclear

localization and is therefore involved in the transcription of genes for antioxidant

synthesizing enzymes.

APAP	-	-	+	+	-	-
Menadione	-	-	-	-	+	+
UO126	-	+	-	+	-	+
Time	4 hrs	1 hrs	1 hrs	1 hrs	4 hr	4 hrs
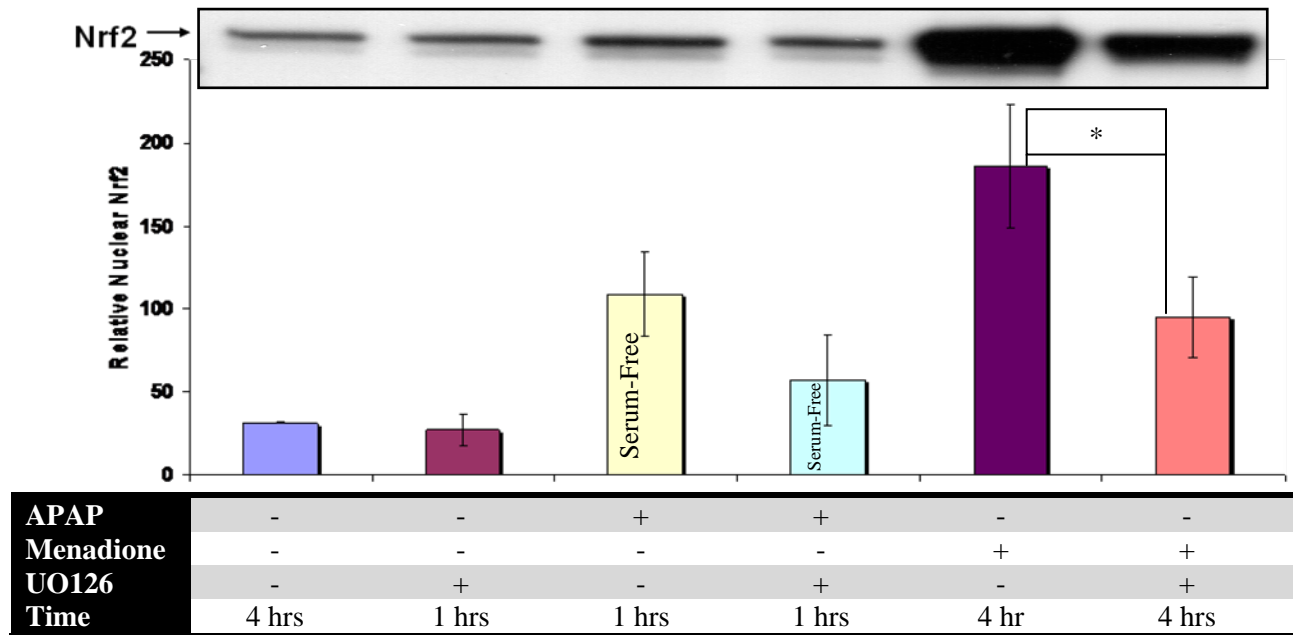
Graph 2. **Testing MAP Kinase Inhibitor.** Shows that the MAPK Kinase family does induce Nrf2 nuclear localization and is therefore involved in the transcription of genes for antioxidant synthesizing enzymes. *shows values that are statistically significant by the p tests with $p<0.05$.

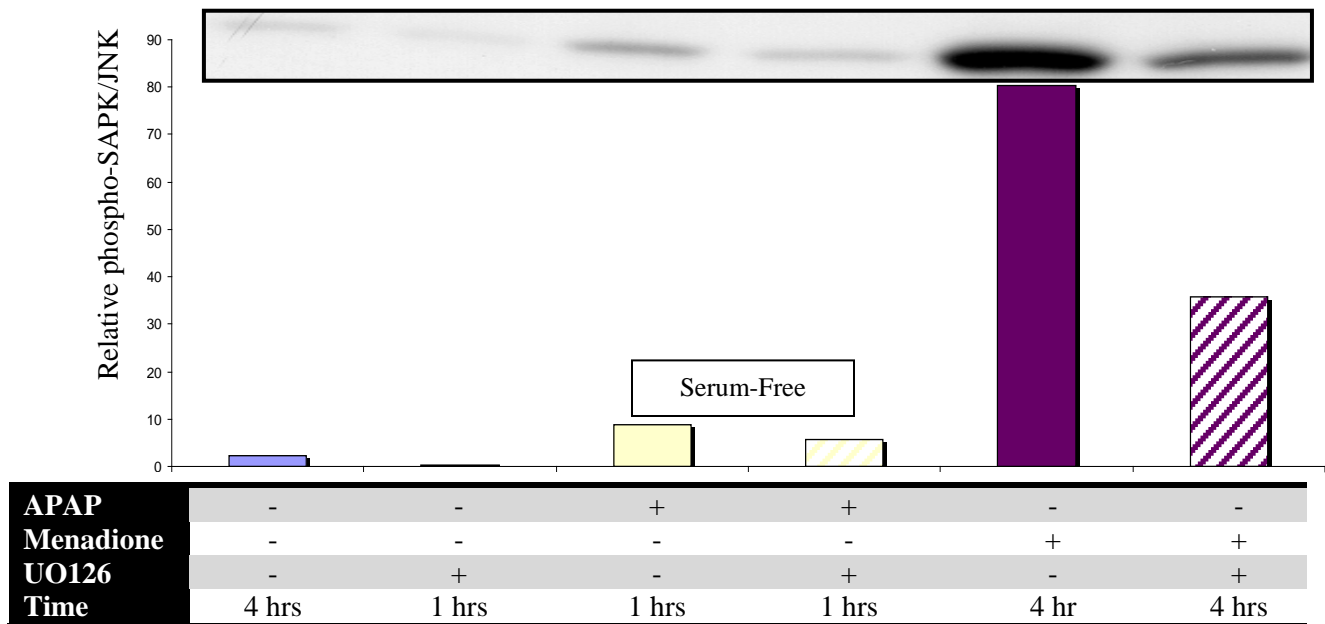### *Confirming MAP Kinase Family Inhibitor*

Further experiments were done to confirm that the UO126, MAPK Kinase family

inhibitor is indeed inhibiting the kinases in its family. To test this, the membrane was re-

probed for these specific kinases: ERK1/2, SAPK/JNK, and p38 MAPK, in their

phosphorylated states. The ERK1/2 data is shown in the next western blot, Graph 3.
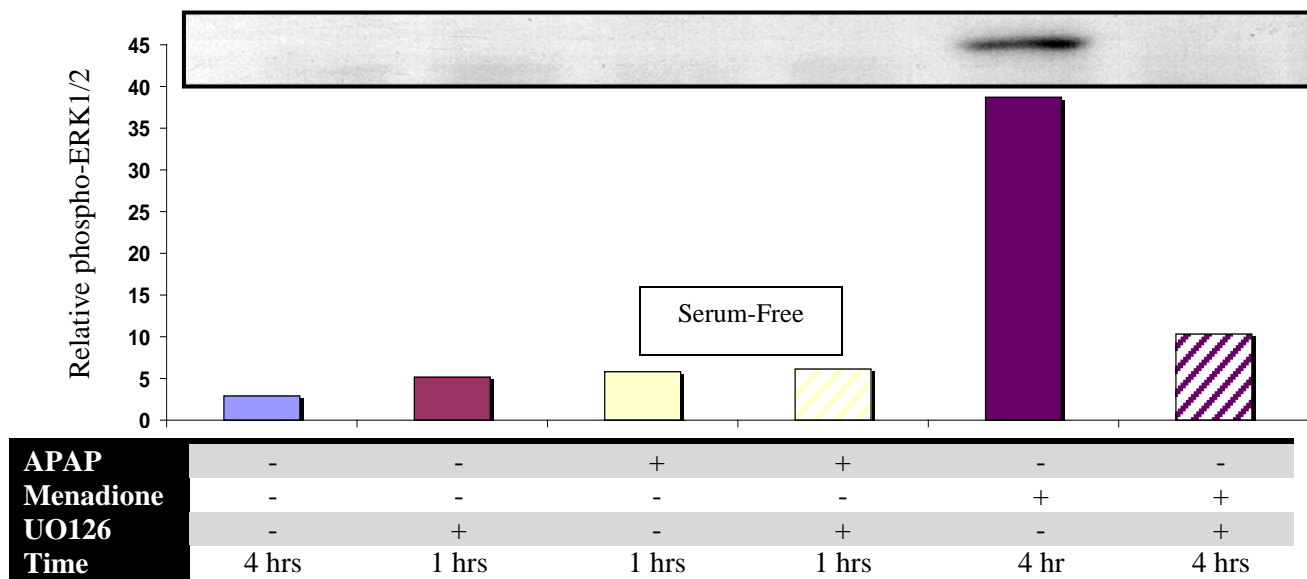
Graph 3. **UO126 inhibits the phosphorylation of ERK Kinase** Erk1/2 is in the MAPK Kinase family and UO126 is confirmed by the western blot above to inhibit its activity.

Shown in Graph 3 is the amount of ERK1/2 Kinase in the cytoplasm. The vehicle with no treatment is low as expected. APAP 1 hr serum-free with and without UO126 shows no change in ERK1/2, and the menadione 4 hrs in serum vs. menadione 4 hrs in serum with UO126 also shows a decrease. This shows that UO126 does indeed inhibit the production of ERK1/2 Kinase and that APAP does not induce ERK1/2 activity.

Graph 4 shows the amount of SAPK/JNK Kinase in the cytoplasm. The vehicle with no treatment and with UO126 is low because there are no inducers. The APAP 1 hr serum-free with and without UO126 shows no change in SAPL/JNK Kinase, and the menadione 4 hrs in serum vs. menadione 4 hrs in serum with UO126 shows a decrease, which shows that UO126 does indeed inhibit the activation of SAPK/JNK Kinase and APAP does not induce its activity.

Graph 4. **UO126 does indeed inhibit the phosphorylation of SAP/JNK**. SAP/JNK is in the MAPK Kinase family and UO126 is confirmed by the western blot above to inhibit its activity.

Shown in Graph 5 is the amount of p38 MAPK in the cytoplasm. The APAP 1 hr serum-free vs. with UO126 does show a decrease in nuclear p38 MAPK, and the menadione 4 hrs in serum vs. menadione 4 hrs in serum with UO126 shows a decrease, which would indicate that UO126 does indeed inhibit the activation of p38 MAPK but the nuclear p38 MAPK levels of the vehicle are unexpectedly high. Our results for p38 is, therefore, inconclusive and we did not pursue study of this kinase any further.

APAP	-	-	+	+	-	-
Menadione	-	-	-	-	+	+
UO126	-	+	-	+	-	+
Time	4 hrs	1 hrs	1 hrs	1 hrs	4 hr	4 hrs
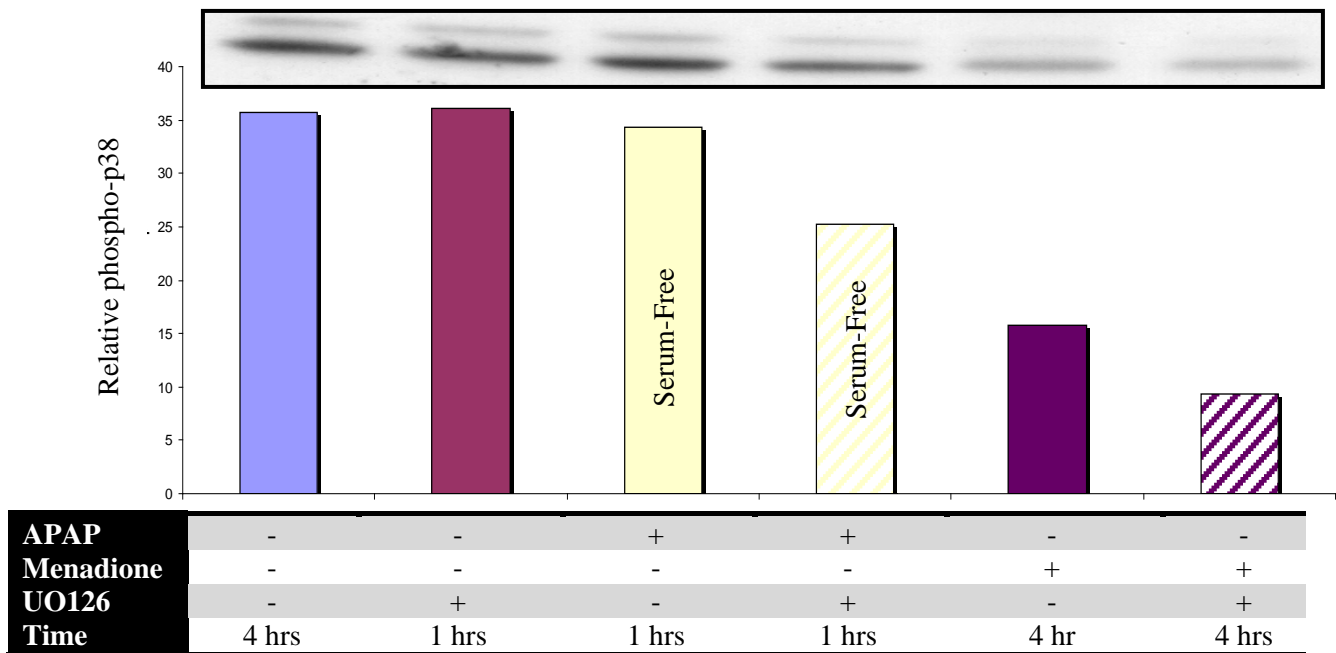
Graph 5. **UO126 does indeed inhibit the phosphorylation of p38**. P38 is in the MAP Kinase family and UO126 is confirmed by the western blot above to inhibit its activity.

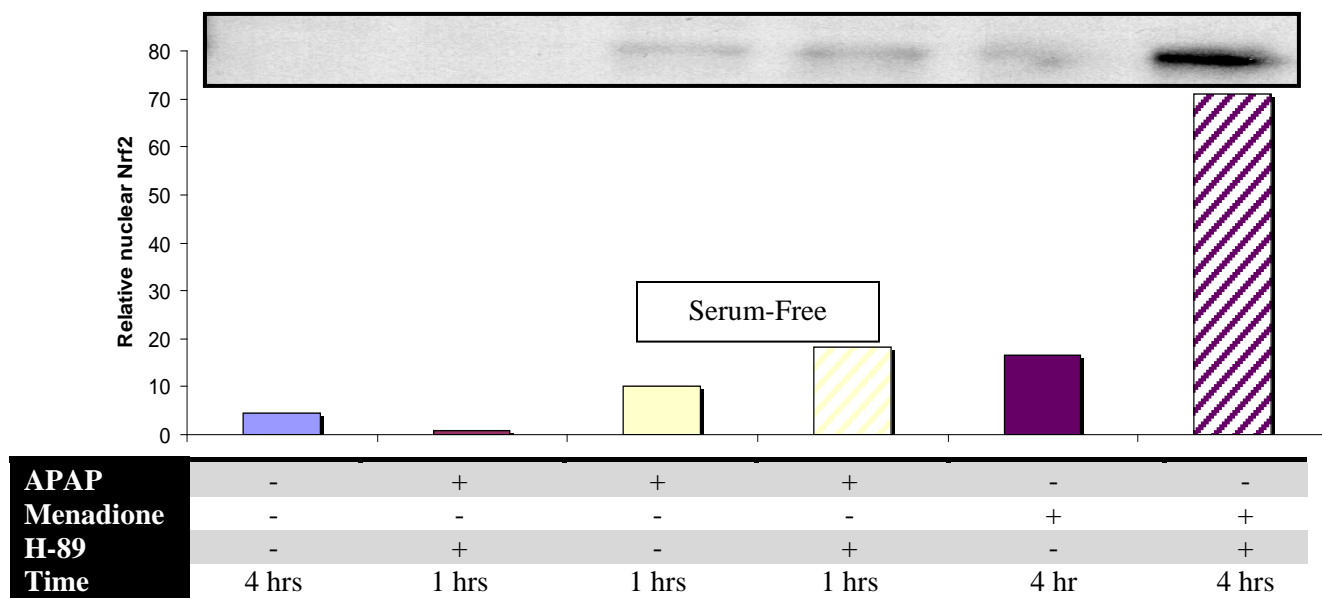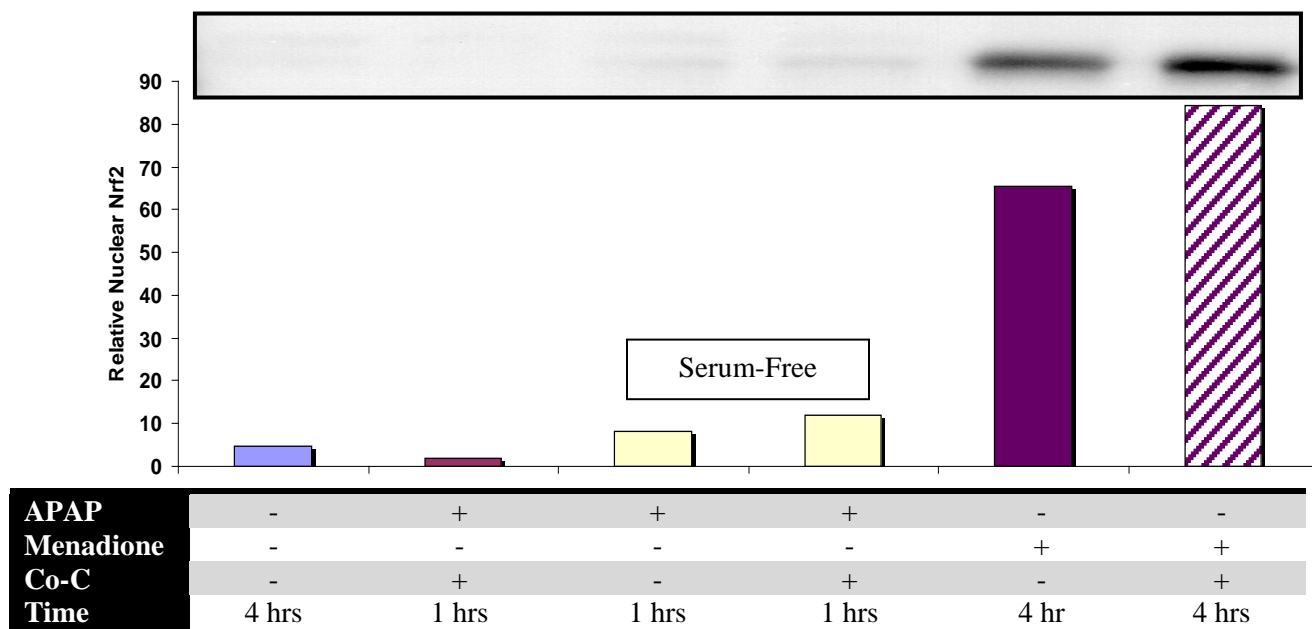### *Negative Controls*

The following two western blots presented in Graph 6 and Graph 7 are negative

controls in order to confirm that the decrease in nuclear Nrf2 was due to the significance of

the MAPK family rather than an artifact. This is done by addition of two other kinase

inhibitors, studying their effect of nuclear Nrf2. Graph 6 shows the western blot with the

addition of a PKA inhibitor (H-89), and Graph 7 is with the AMP Kinase inhibitor

(Compound C) to test conditions. From the data, there is no decrease of nuclear Nrf2 from

the regular condition vs. with the inhibitors. This confirms that the decrease in Nrf2 is due to

the significance of the MAP Kinase family in the Nrf2 signal transduction pathway of stress

response, although it is interesting to note that the inhibition of PKA actually increased

nuclear Nrf2 localization.

APAP	-	+	+	+	-	-
Menadione	-	-	-	-	+	+
H-89	-	+	-	+	-	+
Time	4 hrs	1 hrs	1 hrs	1 hrs	4 hr	4 hrs

Graph 6. **Negative Control with H-89.** Western blot with the addition of a PKA inhibitor shows a decrease in nuclear Nrf2 localization.

APAP	-	+	+	+	-	-
Menadione	-	-	-	-	+	+
Co-C	-	+	-	+	-	+
Time	4 hrs	1 hrs	1 hrs	1 hrs	4 hr	4 hrs

Graph 7. **Negative Control with Co-C.** Western blot with the addition of a AMP Kinase inhibitor, shows no decrease in nuclear Nrf2 localization

EMSA-Lightshift

In order to confirm that Nrf2, once it enters the nucleus, does indeed bind to the ARE, we ran the electrophoretic mobility shift assay. (EMSA) This is important because previous western blots have only indicated increase levels of nuclear Nrf2 but not what it is doing once it enters the nucleus, it could be floating in the nucleus, or binding to a different promoter or other proteins. The EMSA confirms that it is indeed binding to the ARE. This can be seen because DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide electrophoresis; therefore on a gel, DNA complexes will be higher up than free DNA. An EMSA light shift will indicate if a protein in binding to the ARE and an EMSA super shift will tell us which protein is binding to the ARE, this is depicted in Figure 7. The data for the EMSA lightshift is shown is Graph 8.
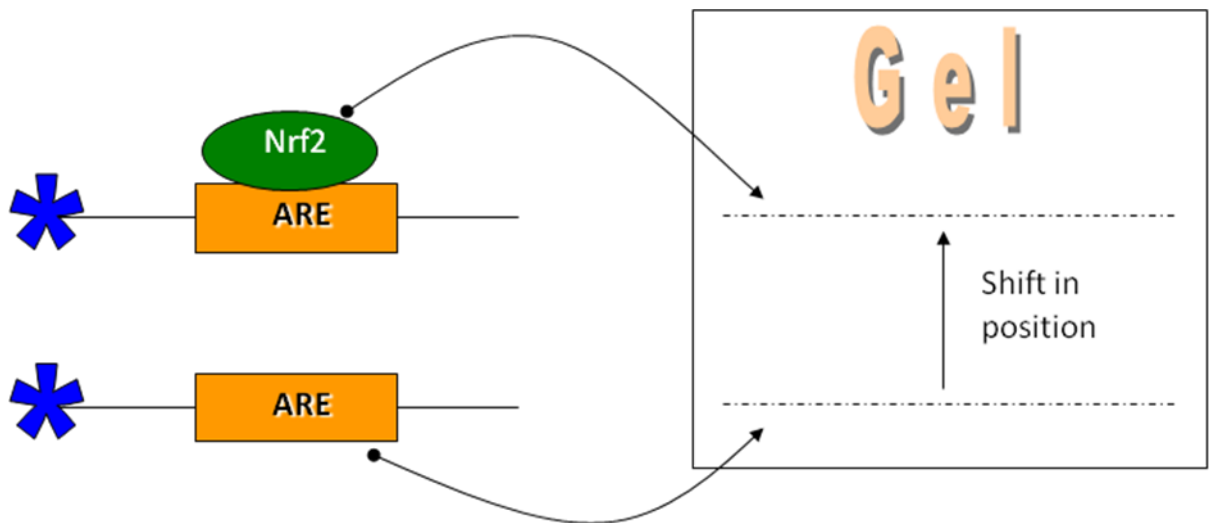

Figure 7. **Concept of EMSA light shift.** DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide electrophoresis; therefore, on a gel, DNA complexes will be higher up than free DNA.
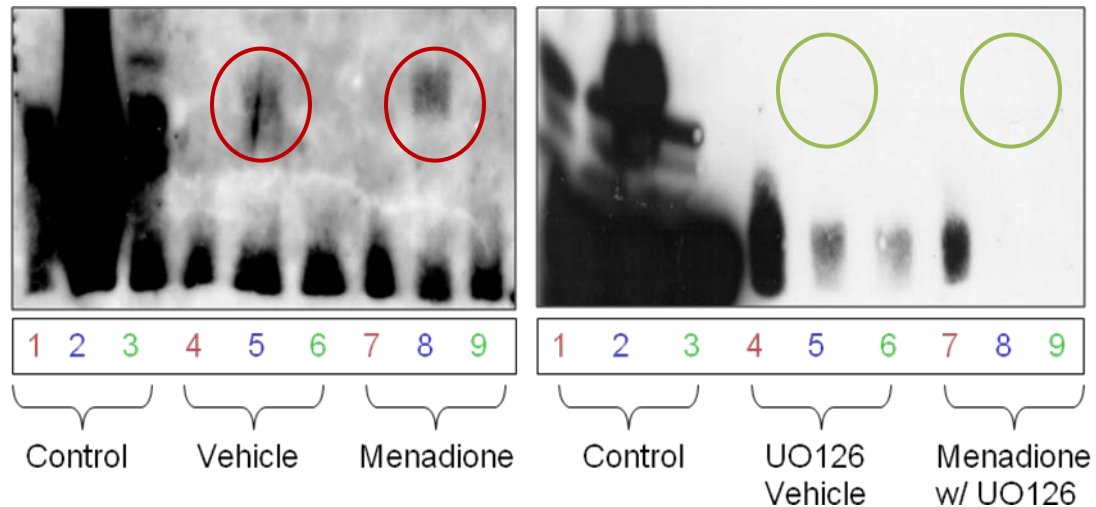
Figure 8. **EMSA Lightshift.** Indicate that a protein in the nuclear extract is binding to the ARE (red circles) and this is lost with the treatment of UO126 (green circles).

For each sample, there are three lanes: the first lane has biotin-ARE DNA, the second lane has biotin-ARE DNA and protein sample, and the third lane has biotin-ARE DNA plus non-biotin-ARE DNA as competitor and protein sample. We chose to use just menadione because we saw that although APAP can also induce Nrf2 nuclear localization, only menadione and not APAP activated ERK1/2 and JNK/SAPK. From Figure 6, the vehicle and the menadione treatment shows a band higher up than the base line (shown in red circle in the left blot), this is not seen when the same cells were treated with UO126 (shown in red circles in the right blot). This data indicates that DNA is complexed with another protein because DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide electrophoresis and will be higher up on the gel than free DNA.

EMSA-Supershift

Lightshift data shows that a protein is binding to the promoter element on the DNA for antioxidant synthesizing enzymes although it does not indicate which protein. This

information can be obtained from a super shift that provides information as to what protein is

binding to the ARE through additional shift in position upon an Nrf2 antibody binding to our

Nrf2 that is bound to the ARE, shown in Figure 9 below.

1. Biotin-ARE
2. Biotin-ARE and protein
3. Biotin-ARE, protein and Nrf2 antibody
4. Biotin-ARE and Nrf2 antibody
5. Biotin-ARE
6. Biotin-ARE and protein
7. Biotin-ARE, protein and Nrf2 antibody
8. Biotin-ARE and Nrf2 antibody
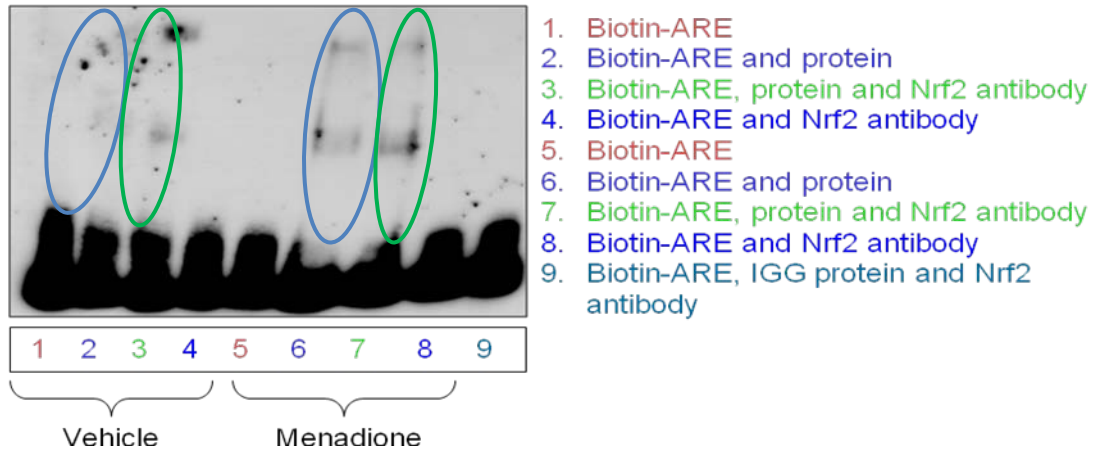9. Biotin-ARE, IGG protein and Nrf2 antibody

Figure 9. **EMSA Supershift.** Confirms that a protein in the nuclear extract is binding to

the ARE although it does not clearly indicate this is Nrf2 due to the noise level of the blots

For super shift, Nrf2 antibodies were added and were expected to bind to Nrf2 protein

which results in a further shift because it is now a bigger complex with the antibody. The

EMSA supershift confirms that a protein in the nuclear extract is binding to the ARE

although it does not clearly indicate this is Nrf2 due to the noise level of the blots.

Although the EMSA does not indicate this, previous western blots probed for Nrf2 do

indicate an increase in nuclear levels and this nuclear level is inhibited with the addition of

UO126. The EMSA shows that with the treatment, there is an increase in the binding to the

ARE that is lost with the addition of UO126. From this information, we can hypothesize that

the protein that is binding to the ARE is indeed Nrf2, which has shown an increase with

inducers and decreased levels with UO126 treatments.

Conclusion and Future Direction

Data presented in this thesis have shown that the MAP Kinase family is involved in the phosphorylation of Nrf2 thus allowing it to enter the nucleus. The EMSA has confirms that Nrf2 is the protein that is entering the nucleus and is likely binding to the ARE, the promoter region for genes of key antioxidant enzymes. This allows the deduction that the MAP Kinase family activates the transcription of key antioxidant enzymes by inducing Nrf2 nuclear localization.

In order to confirm the data that we have obtained, many more replicates must be done as well as a more conclusive supershift to show that the protein that is binding to the ARE is definitely Nrf2. Further tests, such as inhibition with SiRNA, must be done to see if the protein in the MAP Kinase family involved in the activation of Nrf2 is either ERK1/2 or SAPK/JNK. We are particularly interested in ERK because it has been shown to have lower activity in cells from aged animals as compared to young animals. If this kinase phosphorylates Nrf2, it would help to explain the age-related loss in Nrf2 transcriptional activity. Another study would be to investigate the interesting observation that H-89, which inhibits PKA, increases menadione-induced nuclear localization of Nrf2.